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14. ABSTRACT
Sex hormone-binding globulin (SHBG) is a plasma protein that binds androgens, and it acts as a transducer of androgen signaling at the plasma membrane of prostate cancer cells. The human prostate cancer cell line, LNCaP, in addition to having a receptor for SHBG (RSHBG), produces its own SHBG. We devised an inducible system to study SHBG protein expression in LNCaP cells, and expressed tagged versions of SHBG in LNCaP cells, that will also be useful for future studies on its biologic function. We present a complex and novel picture of extrahepatic human SHBG gene expression, including 21 different SHBG gene transcripts generated by alternative splicing, 17 of which are newly described, and the use of three independent SHBG gene promoters, including a novel promoter, PN. We used microarray technology to investigate the effect of SHBG on the DHT response of LNCaP cells, and found evidence for RSHBG signaling and SHBG modulation of AR activity. Further extended studies of the role of SHBG in prostate signaling and the modulation of SHBG expression in prostate cancer are warranted.

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Final Report: "Unique Approaches to Androgen Effects on Prostate Cancer"

INTRODUCTION

Background: Androgens are central both to inception and progression of prostate cancer. Individuals deprived of androgens early in life, such as in castration in youth or in the syndrome of 5α reductase deficiency, do not get prostate cancer. Further, after prostate cancer is established, androgen deprivation causes temporary remission/improvement in the majority of patients. These two clinical facts are not fully explained by our current understanding of how androgens exert their effects.

We have shown the plasma protein sex hormone-binding globulin (SHBG) not only binds testosterone (T) and other androgens in plasma, but is part of a prostatic androgen signal transduction system that starts with a receptor (R_{SHBG}) for SHBG on prostate cell membranes (1). The SHBG-R_{SHBG} complex is activated by an appropriate steroid hormone, such as estradiol (E_2) or 5α-androstan-3α, 17β-diol (3αdiol) (forming the new complex, 3αdiol-SHBG-R_{SHBG}) that triggers a second messenger system, via cAMP. Furthermore, in whole non-cancerous explants of human prostate, the system can cause increases in the secretion of prostate specific antigen (PSA) (2), an event previously thought to be related only to activation of the AR by T or dihydrotestosterone. Our general understanding of the system was based on the assumption that SHBG (which, like most plasma proteins, is produced in the liver) arrived at the prostate only by way of the plasma. We now have shown that prostate cancer cells stain with anti-SHBG antibodies and, more importantly, that a number of prostate cancer cell lines (LNCaP, PC-3, and DU-145) contain both SHBG mRNA and SHBG protein (3). The expression of SHBG by prostate cancer cells raises the important question of how local regulation of SHBG synthesis might function either to act on the sequestration of steroid hormones within the prostate or to alter androgen induced signal transduction in an autocrine or paracrine fashion.

Objective Hypothesis: We propose that the expression of SHBG by prostate cancer cells is biologically regulated and that this SHBG functions to alter the effects of androgens and estrogens within the prostate cancer cell.

Specific Aims: (Aim 1) Generate prostate cancer cell lines that stably express SHBG in a regulatable fashion. Examine (Aim 2) autocrine and (Aim 3) paracrine effects of prostate SHBG synthesis on steroid signaling. (Aim 4) Examine the effect of SHBG synthesis within the prostate on the growth of prostate cancer cell lines both in the presence and absence of androgen.

Experimental Design: We will undertake genetically and pharmacologically based studies to address our hypothesis. Prostate cancer cells from Aim 1 will be exposed to steroids that selectively stimulate either the androgen receptor or the SHBG-receptor based pathways. Further, although AR and SHBG both are high affinity binders of testosterone and DHT, there are other ligands that are specific to each. The same situation exists for

inhibitors of each of the two systems. We will independently stimulate and/or inhibit each of these two signaling systems with such ligands. This pharmacologic approach will allow us to dissect the influence of androgens on these two pathways and further ascertain how each contributes to the growth of prostate cancer cells.

BODY

1. Anti-human SHBG antibodies for Western blot analysis.

A major technical problem that we addressed was identifying an antibody that would be suitable for Western blot analysis of cellular extracts. First, we tested all polyclonal and monoclonal antibodies that we have generated in house or have obtained from outside sources on HepG2 cellular extracts and purified SHBG protein from human serum. None proved useful for Western blot analysis. We next had custom polyclonal antisera generated against both the secreted form of SHBG (encoded by the cDNA used in these studies) and the alternative form of SHBG first described in the testis (2). Unique peptides, CLRPVLPTQSA and CFSLRLTHPPRTW, corresponding to the respective SHBG isoforms, were synthesized and used to immunize rabbits. Affinity purified antisera were positive by ELISA assay, however they did not prove useful for Western blot analysis. Fortunately, in 2004, we obtained a polyclonal antibody, WAK-S102-12-53 (WAK-Chemie, Steinbach, Germany), which is useful for Western blot analysis. As detailed below, we used WAK-S102-12-53 to confirm ELISA and PCR results on cell lines that inducibly or constitutively express various SHBG constructs at elevated levels.

2. Generation of LNCaP clonal cell lines that constitutively overexpress SHBG: pSHBG-FL and pSHBG-MP.

Along with the inducible LNCaP cell line, L5S2 that expresses and secretes abundant amounts of SHBG when treated with the inducing agent, PonA (see **Figure 1**), we synthesized two additional constructs for evaluating the effects of constitutively expressed SHBG in LNCaP cells. The vector used for these experiments was pCMVFlag, a plasmid containing a CMV promoter upstream of an ATG start codon, immediately followed by three iterations of a Flag tag (4) sequence. We generated, 1) pSHBG-FL, a plasmid containing the full length SHBG cDNA sequence cloned immediately downstream of the Flag tags and in the same reading frame, and 2) pSHBG-MP, a plasmid containing SHBG cDNA lacking the 29 amino acid amino terminal leader sequence which, in the liver, is cleaved from the nascent protein before secretion. Thus, this Flag tagged protein mimics the mature, processed SHBG found in serum. However, since it lacks the leader sequence, we hypothesized that the SHBG-MP protein would remain inside cells. We expected that the Flag tag would allow us to specifically detect the expressed pSHBG-FL and pSHBG-MP proteins in western blots. In addition, the Flag tag would produce different sized SHBG proteins, distinguishable from endogenous SHBG.

We expected that SHBG-FL might be detectable in cells, but it would have the Flag tag cleaved along with the signal peptide prior to secretion. However, if the cleavage system

was overloaded with SHBG-FL protein, we might also detect residual full length SHBG-FL protein. We expected that the SHBG-MP construct would remain inside cells because it lacked a leader sequence, and might serve to mimic the fate of absorbed SHBG from outside. The CMV promoter would direct constitutive expression of these proteins at elevated levels.

pSHBG-FL and pSHBG-MP were transfected into LNCaP cells. Following selection, 12 resistant colonies from each transfection were isolated and expanded. LNCaP-FL7 and LNCaP-MP8 were positive in Western blot analysis using anti-Flag monoclonal antibody and the anti-SHBG WAK-S102-12-53 polyclonal antibody (**Figure 2**). LNCaP-FL7 secreted non Flag-tagged SHBG, demonstrating that the leader peptide is properly processed from Flag-tagged SHBG-FL, and that the mature processed protein is secreted just like the secreted form of SHBG in the liver. These results were confirmed by immunofluorescence staining and ELISA assays (data not shown). It is currently unclear why the full length SHBG cDNA, when expressed in LNCaP cells either constitutively or inducibly, is seen as two SHBG bands, though this is likely due to differential glycosylation of SHBG itself.

3. Immunohistochemical analysis of SHBG protein expression in LNCaP cells and effects of steroid binding on SHBG localization.

Previously, we showed that LNCaP cells can be stained using anti-SHBG antibodies (7). We performed similar experiments on inducible L5S2 cells that had been stably transfected with a PonA inducible full length construct. **Figure 3** shows relative levels of SHBG in uninduced vs. induced cells, and the effects of DHT and E2 treatment on SHBG expression. We should point out that the photomigrographs shown in Figure 3 did not reproduce very well for technical reasons. From our observations, there is more pronounced staining of SHBG in the induced L5S2 cells, and pronounced staining within what appears to be the ER and/or Golgi, although this needs to be further investigated. When 2nM DHT is incubated with induced L5S2 cells for 2 or 24 hours, the cells show a rounder morphology, with slightly more focal staining. When treated with 10nM DHT, the intensity of SHBG staining in induced L5S2 cells diminishes and becomes even more focal. Induced L5S2 cells treated with E2 do not appear to show much of a staining difference when compared to untreated induced cells.

4. Characterization of SHBG gene expression in LNCaP and MCF-7 cells- expression is more complex than previously thought

The LNCaP prostate and MCF-7 breast cancer cell lines are known to express SHBG at the mRNA and protein levels and to possess R_{SHBG} activity, making them attractive in vitro models for studying the effects of local SHBG expression on steroid signaling and R_{SHBG}. These cell lines retain both alleles of the p53 gene, located ~35kb away from the SHBG gene locus on chromosome 17p13.1, and due to this extremely close linkage, we presume that both SHBG alleles also remain intact. Initially, we set out to confirm prior reports of SHBG mRNA expression in these cell lines as a prelude to investigating the function of locally expressed SHBG in the prostate and breast (**Figure 4**). As positive

controls for SHBG mRNA expression, we included the human HepG2 liver cell cancer line, known to express abundant amounts of the SHBG/ABP transcript, and normal testis tissue, known to express abundant amounts of the major P_T-derived transcript. However, it soon became apparent that the actual SHBG transcriptional profiles for LNCaP, MCF-7, and the controls are far more complex than has been reported.

Using an oligo-dT primer, a strategy for preferentially generating first strand SHBG cDNAs originating from the poly A tail (the longest run of A residues within the human SHBG coding region is three), first strand cDNAs were synthesized from LNCaP, MCF-7 and HepG2 cells, and from normal testis tissue. As a control for the initial SHBG RT-PCR assays, first strand cDNA was also synthesized from, MCF-7-TF, a MCF-7 derivative cell line that was stably transfected with an SHBG cDNA construct that constitutively overexpresses a transcript containing the human SHBG/ABP coding region.

A previously designed human SHBG RT-PCR assay, spanning exons 5 through 8, was used to generate partial SHBG mRNA expression profiles for LNCaP and MCF-7 cells, and the HepG2, testis and MCF-7-TF controls. Two RT-PCR fragments were anticipated from LNCaP and MCF-7 cells, a contiguous 521nt. fragment that retains exon 7, and a smaller 313nt fragment that lacks exon 7. However, as seen in **Figure 5**, LNCaP and MCF-7 samples gave rise to three distinct RT-PCR fragments. Along with the 521nt and 313nt RT-PCR fragments, whose identities were confirmed by sequencing, an unexpected 176nt RT-PCR fragment of low abundance was also generated. Sequence analysis revealed that this novel 176nt fragment was derived from a transcript(s) in which exon 5 was spliced directly to exon 8, a finding not previously reported. The presence of this same novel 176nt RT-PCR fragment in the HepG2 and normal testis lanes implied that prior reports of human SHBG mRNA expression are incomplete. Further analysis of the HepG2 and normal testis RT-PCR patterns revealed a faint, intermediate RT-PCR fragment of approximately 384nt, which would be the predicted size of an SHBG transcript that lacked only exon 6 sequences. This fragment has repeatedly proven resistant to reamplification and thus remains uncharacterized. As expected, the MCF-7-TF RT-PCR control gave rise to only the contiguous 521nt fragment, since the highly overexpressed full length SHBG/ABP cDNA transcript overwhelms the RT-PCR assay.

The relative intensities of the 521nt. and 313nt bands varied between samples, supporting prior findings that steady state levels of SHBG mRNAs are influenced by tissue specific factors. Whereas LNCaP and MCF-7 had relatively more of the 521nt fragment than the 313nt fragment, HepG2 had 521nt and 313nt fragments of relatively similar intensities, and normal testis tissue generated more of the exon 7-lacking 313nt fragment. Why HepG2 does not express more of the 521nt fragment is unclear, and from data presented below, HepG2 differs from normal liver tissue in this regard.

5. Generation of an overall picture of human SHBG gene transcription using an exon 2-8 RT-PCR assay.

To gain deeper insight into the complexity of SHBG mRNA expression, an exon 2-8 RT-PCR assay was designed to screen for alternative splicing of the downstream exons (3-7) in a single reaction, intentionally bypassing the assessment of individual contributions from P_L and P_T. First strand cDNAs prepared from normal liver, prostate and breast tissues were also included as templates for RT-PCR. Taking into account the results presented in Figure 5, three bands were expected in the exon 2-8 assay, a contiguous 1075nt exon 2-8 RT-PCR fragment, an intermediate 867 nt. fragment derived from SHBG transcripts that lack exon 7, and a small 730 nt. fragment derived from SHBG transcripts that lack exons 6 and 7.

As is shown in **Figure 6** and summarized in **Table 1**, collectively, nine distinct RT-PCR fragments were generated in the exon 2-8 RT-PCR assay. Six of these nine RT-PCR fragments were successfully reamplified and sequenced. The largest, 1075nt RT-PCR fragment, contained the expected contiguous exon 2-8 sequence, while the smaller sized fragments were derived from SHBG transcripts that had undergone alternative splicing of exons, 4, 6, and/or 7. From largest to smallest, RT-PCR fragments were derived from alternatively spliced SHBG transcripts that lacked exon 4 (913nt), exon 7 (867nt), exons 6 and 7 (730nt), exons 4 and 7 (605nt), and exons 4, 6 and 7 (468nt). That exon 4 undergoes alternative splicing was confirmed using an RT-PCR assay that spanned exons 3 and 5 (data not shown). During the course of these studies, an independent group also observed alternative splicing of exon 4, using an RT-PCR assay that included primers specific for exons 3 and 7 (9). The remaining three RT-PCR fragments, which reproducibly appeared in this assay, were resilient to repeated attempts at reamplification, thus their structures remain unknown at the present time. As expected, only a single, intense contiguous exon 2-8 RT-PCR fragment was generated from MCF-7-TF, since the stably transfected cDNA construct is highly expressed relative to the endogenous transcripts.

The exon 2-8 RT-PCR profiles of the HepG2, LNCaP, and MCF-7 cell lines were qualitatively similar, with HepG2 displaying the most intense overall pattern, followed by LNCaP, and then MCF-7. Among normal tissues, the contiguous 1075nt fragment was most abundant in liver, whereas the 867nt fragment lacking exon 7 was most abundant in the testis. Comparing cell lines to their corresponding normal tissues, HepG2 generated less of the contiguous 1075nt fragment, and more of the alternatively spliced fragments than did normal liver. On the other hand, LNCaP and MCF-7 had exon 2-8 RT-PCR profiles that were similar to normal prostate and normal breast, respectively. How SHBG is expressed within individual cell types that comprise the liver, breast, and prostate, and whether the SHBG expression pattern is conserved or is altered in cell lines compared to their specific progenitor cells, requires further study.

6. Identification of transcriptional start sites from P_L and P_T and identification of a novel human SHBG gene promoter, P_N.

As a prelude to designing RT-PCR assays to ascertain the splicing patterns of full length SHBG mRNAs sequences, we addressed whether the human SHBG gene might use previously uncharacterized promoters in addition to P_L and P_T. An active, alternative upstream SHBG promoter (P_A) is present in the rat, and was speculated to be active in humans. We undertook a nonbiased approach to characterize transcriptional start sites with the aim of identifying the previously reported SHBG gene promoters (P_L and P_T), and to determine whether the human homologue of P_A, or any other novel SHBG promoter(s) might be utilized. A modified RACE ("Rapid Amplification of cDNA Ends") assay was performed on RNA isolated from the LNCaP, MCF-7, and HepG2 cell lines and from normal testis tissue. This modified RACE assay included enzymatic steps designed to preferentially prime full length transcripts containing 5' end cap structures, for subsequent PCR amplification. The first PCR step incorporated a reverse SHBG primer specific for exon 3 and a 5' end universal primer, while the second, nested PCR step, designed to generate RACE fragments containing SHBG exon 1 sequences, incorporated a reverse SHBG primer specific for exon 2 and a second, more proximal 5' end universal primer.

LNCaP, MCF-7, HepG2, and normal testis tissue gave rise to multiple RACE fragments (data not shown), each of which was reamplified and sequenced. Four of these fragments were found to be specific for human SHBG mRNA transcripts (the remaining fragments were either not specific for SHBG, or were comprised of SHBG sequences that had recombined with sequences from other chromosomes, and thus were considered to be experimental artifacts). Two distinct exon 1L 5' end boundaries were detected, indicating that P_L derived transcripts initiate at A residues located 79 nt and 38 nt upstream of the exon 1L ATG start codon. This is different from the case of mice that express a human SHBG transgene, where transcriptional initiation occurs at a single point, 60 nt. upstream of the ATG start codon (10), though it is similar to the picture of SHBG transcriptional initiation in the rat, where two start sites have been mapped 36 and 116 nt upstream of the rat SHBG ATG start codon (11,12).

Normal testis tissue produce a RACE fragment containing exon 1T sequences that extended to a G residue, 183 nt. upstream from the splice donor site. Surprisingly, the sequence we obtained for exon 1T had an A residue inserted at position six, as compared to the all previously published sequences, and those present in Genbank. We are in the process of performing further studies to determine whether this is indeed a human polymorphism, or whether this difference so close to the 5' end of the transcript is an artifact of the RACE. If this single A residue insertion does indeed exist, it would create an ATG start codon beginning at position six of P_T derived transcripts, followed by an open reading frame of an additional 39 amino acids, contained entirely within exon 1T.

LNCaP presented a RACE fragment consisting of a novel 164nt sequence spliced directly to SHBG exon 2. This 164nt sequence mapped to an uninterrupted genomic locus

located 17kb upstream of P_L on chromosome 17p13.1, located within the first intron of the FXR2 gene. This novel sequence is referred to herein as SHBG exon 1N (**Figure 7**). We confirmed the 1N transcript initiation site in LNCaP cells in a second RACE assay, using a downstream primer complementary to 3' end sequences within exon 1N and the same 5'adaptor based primer (data not shown). A short exon 1N-2 RT-PCR assay was also performed on LNCaP, MCF-7, and HepG2 cells to confirm that exon 1N is incorporated into mature SHBG mRNA transcripts. A single RT-PCR fragment of expected size was generated from LNCaP and MCF-7, but not from HepG2 (data not shown). Because of the limitations of this RACE strategy, if they exist, additional minor SHBG promoters, and promoters specific for other tissues may have gone undetected in this assay.

7. Characterization and identification of novel human SHBG transcripts extending from P_L, P_T, and P_N using exon 1L-8, exon 1T-8, and exon 1N-8 RT-PCR assays.

Having characterized SHBG exon 1L, 1T, and 1N SHBG sequences, three RT-PCR assays were performed to ascertain the splicing patterns of SHBG transcripts arising from P_L, P_T, and P_N. 1L-8, 1T-8, and 1N-8 assays were designed utilizing a common, downstream exon 8 primer, and upstream primers specific for either exons 1L, 1T, or 1N (**Figure 8,** summarized in **Table 1**). Sequence analysis was performed on all RT-PCR fragments that underwent successful reamplification. For reasons that are unclear, normal prostate and breast gave fewer RT-PCR fragments in the exon 1-8 RT-PCR assays than in the exon 2-8 RT-PCR assay. In addition to generating counterparts of alternatively spliced SHBG transcripts that had previously been characterized in the exon 2-8 RT-PCR assay, novel small sized RT-PCR fragments were generated from samples displaying the highest abundance of exon 1-8 RT-PCR fragments. HepG2, testis, and liver samples generated a 463nt exon 1L-8 RT-PCR fragment, which sequence analysis revealed to be comprised of exon 1L, 2, 3, and 8 sequences. Similarly, testis and liver produced a 484nt exon 1T-8 fragment that consisted of exon 1T, 2, 3, and 8 sequences, and LNCaP gave a faint, exon 1N-8 479nt fragment, that consisted of exon 1N, 2, 3, and 8 sequences. To our knowledge, this is the first account of human SHBG transcripts that lack exon 5 sequences.

The exon 1L-8 and 1T-8 assays revealed that the major SHBG/ABP transcript is present in all samples, and aside from normal breast tissue, so is the major testis transcript. It is noteworthy that normal liver, prostate, and breast appear to express a lower proportion of alternatively spliced SHBG transcripts than do the HepG2, LNCaP, and MCF-7 cancer cell lines. Whether this finding reflects the heterogeneous cellular makeup of tissues, or events related to cellular transformation is unclear at this time.

LNCaP was the predominant expresser of exon 1N transcripts, giving rise to four RT-PCR fragments of relatively high abundance, including the exon 1N-8 transcript, and transcripts lacking exons 7, 4 and 7, and 4, 6, and 7. We note that the HepG2 exon 1N-8 RT-PCR assay did not reproducibly generate the same fragments that appear in Figure 8 (data not shown). We assume that this variation is due to exceedingly low P_N activity in

HepG2 cells, considering that normal liver failed to generate any exon 1N-8 RT-PCR fragments.

8. Quantitative PCR analysis of total overall human SHBG gene expression and expression from each of P_L, P_T, and P_N.

We performed four independent real time, quantitative PCR (qPCR) assays to compare total human SHBG mRNA transcript abundance in LNCaP, MCF-7, and HepG2 cells, and normal prostate, breast, liver, and testis tissues, and to determine relative individual contributions from each of the three promoters, P_L, P_T, and P_N (**Figure 9**). The first qPCR assay was used to quantitate total amounts of SHBG transcripts by using primers specific for exons 2 and 3, common to all of the SHBG transcripts identified in this study. The three exon 1-specific qPCR assays incorporated upstream primers specific for exons 1L, 1T, and 1N, respectively, and exon 2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels, also measured by qPCR, were used to normalize samples.

Among those normal tissues tested, total steady state levels of SHBG mRNA were highest in liver and testis as expected, two orders of magnitude greater than in prostate and tissue (**Figure 9A**). HepG2 cells exhibited a 31-fold lower total steady state SHBG mRNA level than did normal liver tissue. This difference, perhaps coincidentally, is similar to the GAPDH correction factor used to normalize expression between these two samples. LNCaP expressed 1.25-fold more total SHBG mRNA than normal prostate tissue, whereas MCF-7 cells expressed 8.7-fold less than normal breast tissue.

Overall, the three individual exon 1-2 qPCR assays gave results that were comparable to those obtained in the RT-PCR assays (**Figure 9B**). 1L containing transcripts were expressed highest in normal liver tissue, 55-fold more abundant than in normal testis tissue and two orders of magnitude higher than in normal prostate and normal breast tissue. Compared to their respective parental tissues, the three cancer cell lines examined in this study all had a reduced steady state expression of 1L containing transcripts. A 35-fold reduction was observed in HepG2 cells (perhaps due to lower amounts of the SHBG/ABP transcript), and 3-fold and 20-fold reductions were observed in LNCaP and MCF-7 cells, respectively. 1T transcripts were by far most pronounced in normal testis tissue, 165-, 703-, and 1828-fold more abundant than in normal liver, prostate and breast tissue, respectively. 1T transcripts were 5-fold more abundant in LNCaP as compared to normal prostate tissue, whereas they were 30-fold less abundant in HepG2 than in normal liver tissue and 12-fold less abundant in MCF-7 than in normal breast tissue. 1N transcripts were most abundant in normal prostate tissue, exhibiting 4-fold higher steady state expression levels than normal breast and normal testis tissue. 1N transcripts were undetectable in normal liver tissue (no evidence of expression after 45 cycles of PCR). LNCaP cells exhibited twice the expression of 1N transcripts as normal prostate tissue, whereas MCF-7 cells exhibited 70% expression compared to normal breast tissue. As was the case for RT-PCR, 1N transcripts were barely detectable in HepG2 cells.

9. Regulation of gene expression in LNCaP cells by SHBG- microarray analysis.

Another major focus was to determine whether SHBG expression can affect overall gene expression in LNCaP cells. In our initial proposal, we outlined a series of reporter assays that would be used to determine the effects of SHBG expression on a few androgen responsive genes and constructs that were sensitive to PKA activation. The availability of microarray technology at rapidly falling costs, made microarray analysis a preferable approach for determining the effects of SHBG on gene transcription. As mentioned above, we generated an LNCaP cell line, L5S2, that expresses and secretes abundant amounts of SHBG when treated with the inducing agent, PonA (Figure 1). We also generated a sister cell line, L5V4, a vector control that lacks the SHBG insert.

L5S2 and L5V4 cells were seeded in medium containing 10% charcoal stripped fetal calf serum. Plated cells were divided into two groups, one treated with the inducing agent, PonA (10uM), and the other treated with the carrier ethanol, for 24 hr. Triplicate wells from each group of cells were then treated for an additional four, eight, 12, 24, and 48 hours with DHT.

This strategy gave us the following treatment conditions (each condition was performed in triplicate)-

1. L5S2 negative treatment control (carrier treated only)*
2. L5S2 PonA treated alone*
3. L5S2 PonA treated, DHT treated 4 hours*
4. L5S2 PonA treated, DHT treated 8 hours
5. L5S2 PonA treated, DHT treated 12 hours
6. L5S2 PonA treated, DHT treated 24 hours*
7. L5S2 PonA treated, DHT treated 48 hours

8. L5V4 negative treatment control (carrier treated only)*
9. L5V4 PonA treated alone*
10. L5V4 PonA treated, DHT treated 4 hours*
11. L5V4 PonA treated, DHT treated 8 hours
12. L5V4 PonA treated, DHT treated 12 hours
13. L5V4 PonA treated, DHT treated 24 hours*
14. L5V4 PonA treated, DHT treated 48 hours

Total cellular RNA was prepared from each of the above triplicate samples.

Due to the expense of microarray analysis, we chose to analyze only the triplicate samples from each of the treatment conditions denoted above with a "*". The data that will be presented here are from single samples only, the remaining two samples from each set were recently hybridized to gene chips, and those data are currently being processed.

In addition to LNCaP, we analyzed single samples from HepG2 liver cancer cells and a constitutive SHBG overexpressing clone, HepG2myc23, as well as from MCF-7 breast cancer cells, a constitutive SHBG overexpressing clone, MCF-7myc23, and the same two cell lines treated with 10nM estradiol for 4 or 24 hours. Each total cellular RNA sample was pretreated with RNase-free DNase. Total cellular RNAs were sent to our colleagues at the Yerkes Genomics Core Facility at Emory University. RNAs were analyzed by Agilent Bioanalyzer to check the RNA qualities, all of which passed stringent controls. Samples were labeled and hybridized to Affymetrix Human Genome U133 Plus 2.0 Array chips. Raw data was collected by GCOS software and analysis of the microarray data was performed using GeneSpring software. The quality of data, as measured by internal controls and housekeeping genes, was excellent.

A general view of the scope of this work is presented in **Figure 10**, which shows the relationship between the 16 samples based on their gene profiles. As expected, there are clusters between the LNCaP, HepG2, and MCF-7 cells. Furthermore, L5S2 samples cluster together, as do L5V4 samples. Similarly, the constitutive SHBG expressing MCF-7myc23 samples cluster together, as do the parental MCF-7 samples.

We are currently performing a detailed analysis of the expression data to determine which genes are specifically regulated by SHBG alone, and by activation of the steroid-SHBG-R_{SHBG} signaling pathway. In **Figures 11A-C**, we present an analysis of our raw data on single gene chips only, showing those genes whose expression displays the greatest induction, or repression in L5S2 cells compared to L5V4 vector control cells treated with PonA alone for 24 hours, PonA and then DHT for 4 hours, and Pon A and then DHT for 24 hours. The analysis of the triplicate chips is currently being performed. Again, the quality of data, as measured by internal controls and housekeeping genes, was excellent.

L5S2 cells induced with 10uM PonA for 24 hours had a 212-fold increase in SHBG expression compared to similarly treated L5V4 cells. They also exhibited a 50- to 100-fold overexpression of 10 genes, one of which is a myosin light chain kinase, while the nine others have not been functionally identified. Many of the most repressed genes appear to be involved in various metabolic processes, e.g. a glutamatekainate receptor subunit, so the authenticity of these genes' reduced expression needs to be further established.

L5S4 cells induced with 10uM PonA for 24 hours and then treated with 10nM DHT for an additional 4 hours had a 608-fold increase in SHBG expression compared to similarly treated L5V4 cells. They also exhibited a 50- to 281-fold overexpression of 15 genes, including contactin 1, a growth differentiation factor, and 13 genes of unknown function. The most repressed genes also appear to be involved mostly in metabolic processes.

L5S4 cells induced with 10uM PonA for 24 hours and then treated with 10 nM DHT for an additional 24 hours had a 394-fold increase in SHBG expression compared to similarly treated L5V4 cells. They also exhibited a 50- to 266-fold overexpression of 6 genes, including an endothelian receptor, a melanoma associated gene, and four genes of

unknown function. As above, the most repressed genes appear to be involved mostly in metabolic processes.

10. Generation of LNCaP clonal cell lines displaying reduced SHBG expression using RNAi technology.

RNAi technology has rapidly established itself as a powerful method for the downregulation of gene expression since we first submitted this application. We have, in the last year, turned to this technology to silence SHBG gene expression in LNCaP cells, with the goal of establishing clonal cell lines that have downregulated SHBG expression, and using these cells to complement our gene expression studies described above. Two shRNA silencing retroviral constructs specific for human SHBG were purchased from Open Biosystems (Huntsville, Alabama), along with a nonsilencing control construct. These constructs were transfected twice in parallel into the LinX amphotropic packaging cell line, and pools of puromycin resistant colonies were obtained. Supernatants containing infectious retroviral particles were isolated and filtered, and assayed on Rat6 fibroblasts. Each retroviral supernatant was able to induce puromycin resistant Rat6 colonies in titres of between 10-100/ml, in line with the manufacturer's specifications. Each infectious retroviral pool has been used to infect LNCaP cells, as well as HepG2 cells as a control. Pools of 10 or more puromycin resistant LNCaP cells were generated from each of the two shRNA constructs and from the nonsilencing control. We are presently in the process of assessing the degree of SHBG downregulation in the pools derived from the SHBG shRNA construct. If these LNCaP SHBG shRNA infectant pools display downregulation compared to control infected LNCaP cells, they will be assayed by microarray as above to determine whether specific genes that appear to respond to SHBG and SHBG plus DHT display opposite effects when SHBG is downregulated.

KEY RESEARCH ACCOMPLISHMENTS

1. Identification of the anti-human SHBG polyclonal antibody, WAK-S102-12-53, for use in Western blot analysis.
2. Synthesis of pSHBG-FL and pSHBG-MP, flag tagged full length and mature processed SHBG constructs for constitutive expression. Generation of candidate LNCaP-FL7 and LNCaP-MP8 clonal cell lines that constitutively expresses stably incorporated flag tagged full length and mature processed SHBG construct, respectively.
3. Demonstration that elevated amounts of SHBG can be made in LNCaP cells, and that LNCaP overexpressing cells secrete SHBG. Demonstration that the flag tagged mature processed SHBG protein is stable and remains intracellular.
4. Discovered that the human SHBG gene utilizes three promoters, including a novel promoter, P_N, located within intron 1 of the FXR2 gene, ~17kb away on chromosome 17p13.1
5. Human exon 1N is 164 nt. long, and contains a very short open reading frame of only 3 amino acids. Exon 1N shows homology to other mammalian species, however the ATG start site is not well conserved.
6. Mapped the transcriptional start sites for human SHBG exon 1L. Human exon 1L transcripts initiate at 2 transcriptional start sites, which extend 38 and 79 nt. upstream of the exon 1L ATG start codon. This is similar to the rat, which also has been shown to possess two exon 1L start sites. However, it is different from a transgenic mouse model that expresses a human SHBG gene, where human exon 1L transcripts originate at a single start site, 60nt upstream of the ATG start codon.
7. Mapped the transcriptional start site for human SHBG exon 1T. Exon 1T transcripts were determined to start 183nt upstream of the exon 1T splice donor site. Interestingly, we discovered an "A" insertion at position 6 of exon 1T, this creates an putative ATG start codon. We are in the process of confirming these results, and determining whether this constitutes a SNP in humans.
8. Discovered that, due to alternative splicing, the human SHBG gene expresses up to 21 transcripts, 17 of which are novel (**Figure 12**). During this study, another group reported alternative splicing of exon 4, in addition, we found alternative splicing of exons 5 and 6, in addition to early reports of alternative splicing of exon 7.
9. Suggest a model in which SHBG transcripts containing exon 1L may encode stable SHBG isoforms, those that retain the exon 8 reading frame. This includes transcripts that have undergone splicing of exon 4, exons 6 and 7, and exons 4, 6, and 7. Indeed, the transcript lacking exon 4 may be responsible for the SHBG isoform observed in human sperm. If indeed these isoforms are stably expressed, they will lose residues that contribute to SHBG steroid binding and dimerization.

They will retain the R_{SHBG} binding domain within exon 3, and may therefore affect R_{SHBG} signaling.

10. Quantitated amounts of SHBG transcripts present in prostate, breast, liver and testis, and in the LNCaP, MCF-7, and HepG2 cell lines. We broke down SHBG expression into its three different exon 1 components. We showed that the liver expresses the highest amounts of SHBG exon 1L containing transcripts, and the testis expresses the highest amounts of SHBG 1T containing transcripts. We found that LNCaP cells express the highest amounts of exon 1N containing transcripts. Interestingly, in this sample, the cancer cell lines express less of the SHBG/ABP transcript than their parental tissues, suggesting that further study of SHBG downregulation in prostate, breast, and liver cancer is warranted.
11. Our results provide support for a model in which the human SHBG gene has hijacked promoter sequences from its two neighboring 5' end genes (**Figure 13**), and that in mammals, the SHBG gene is still undergoing evolutionary flux.
12. SHBG alone is a potent stimulator/repressor of a number of genes. This new and exciting finding opens up new ways in which to think about how signaling through this pathway occurs.
13. SHBG affects DHT induction of genes in LNCaP cells after both 4 hours and 24 hours of DHT treatment. These result are consistent with our main hypotheses posed at the beginning of these studies, namely that in the prostate, SHBG modulates steroid signaling through R_{SHBG} and that SHBG can act as an intracellular buffer for steroids. We are analyzing our microarray data in detail to determine which genes may be modulated by R_{SHBG}, and which androgen responsive genes may be affected by the presence of SHBG in LNCaP cells.

REPORTABLE OUTCOMES:

1. Fourth International Symposium on Hormonal Carcinogenesis. Valencia, Spain, June 21-25, 2003

Poster presentation-

IMMUNOHISTOCHEMICAL AND *IN SITU* DETECTION OF SEX HORMONE-BINDING GLOBULIN (SHBG) EXPRESSION IN BREAST AND PROSTATE CANCER: IMPLICATIONS FOR HORMONE REGULATION

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Book Chapter-

Kahn SM, Hryb DJ, Nakhla AM, Romas NA, Rosner W**.** Immunohistochemical and *in situ* detection of sex hormone-binding globulin (SHBG) expression in breast and prostate cancer: Implications for hormone regulation. "Hormonal Carcinogenesis, vol. 4." (2005) Jonathan Li, Sara Li, Antonio Llombart-Bosch, editors. Springer, publisher. pp.508-514

1. Third International Meeting- Rapid Responses to Steroid Hormones. Florence, Italy, Sept. 12-14, 2003

Poster presentation-

IMMUNOHISTOCHEMICAL AND *IN SITU* DETECTION OF SEX HORMONE-BINDING GLOBULIN (SHBG) EXPRESSION IN BREAST AND PROSTATE CANCER: IMPLICATIONS FOR HORMONE REGULATION

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3. Era of Hope Conference. Philadelphia, Pa. June 8-11, 2005

Poster Presentation-

HUMAN SEX HORMONE-BINDING GLOBULIN (SHBG) GENE EXPRESSION: UTILIZATION OF MULTIPLE PROMOTERS AND COMPLEX ALTERNATIVE SPLICING OF TRANSCRIPTS

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4. Society for Basic Urologic Research Conference, Miami, Fla. December 1-4, 2005

Poster Presentation-

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5. 31st Annual Meeting of the International Urogynecological Association (IUGA), Athens, Greece, Sept 9th-12, 2006
HUMAN SEX HORMONE-BINDING GLOBULIN (SHBG) GENE EXPRESSION: UTILIZATION OF MULTIPLE PROMOTERS AND COMPLEX ALTERNATIVE SPLICING OF TRANSCRIPTS
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and College of Physicians and Surgeons, Columbia University, New York, N.Y

6. Fifth International Symposium on Hormonal Carcinogenesis. La Grande Motte, France, Sept. 10-13, 2006
Poster Presentation-
SEX HORMONE-BINDING GLOBULIN INFLUENCES GENE EXPRESSION OF LNCaP AND MCF-7 CELLS IN RESPONSE TO ANDROGEN AND ESTROGEN TREATMENT
Scott M. Kahn[1,2] Yu Hua Li [3], Daniel J. Hryb[1,2], Atif M. Nakhla[1], Nicholas A. Romas[1,2], and William Rosner[1]
Departments of Medicine[1] and Urology[2], St. Luke's/Roosevelt Hospital Center, and College of Physicians and Surgeons, Columbia University, New York, N.Y, and Yerkes National Primate Research Center Microarray Core[3], Emory University, Atlanta, Ga.

7. 22nd Annual EAU Congress, Berlin, Germany, March 21-24, 2007
ENDOGENOUSLY EXPRESSED SEX HORMONE-BINDING GLOBULIN (SHBG) MEDIATES THE STEROID RESPONSE OF PROSTATE CANCER CELLS
S. Kahn[1], Y. Li[2], A. Nahkla[1], D. Hryb[1], W. Rosner[1], N. Romas[1]
Columbia University, Urology, New York, United States, (2) Emory University, Yerkes Microarray Core, Atlanta, United States

Pilot Funding Awards-

- "Identification of Androgen Responsive Genes That Control Prostate Cancer Growth Independent of the Androgen Receptor.
  Principal Investigator: Scott M. Kahn, Ph.D
  Agency: St.Luke's Roosevelt Associate Trustees Research Award
  Type:Seed funding 6/06-5/07 $15,000

Effects of SHBG downregulation by RNAi technology on the androgen response of LNCaP cells will be investigated by microarray analysis. Results will be compared to those obtained on similarly treated LNCaP cells that inducibly overexpress SHBG.

- "Modulation of Androgen Responsiveness in Prostate Cancer by Sex Hormone-Binding Globulin"-renewal

Principal Investigator, Scott M. Kahn, Ph.D.

Agency- American Hellenic Educational Progressive Association Cancer Research Foundation Period: 2005-2007, current year- $ seed grant award.

This study is to determine the effects of SHBG on gene expression in prostate cells, and how SHBG modulates androgen signaling.

CONCLUSIONS:

The results from our studies support our original hypothesis that SHBG acts to regulate the DHT response of prostate cells. Because of rapid technologic advances, instead of using reporter systems that assess single downstream responses, we embraced microarray technology, which has enabled us to assess overall gene expression in response to SHBG and DHT in LNCaP cells. We made the interesting and unexpected discovery early on in these studies that expression of SHBG at the mRNA level was not the simple picture that had been reported earlier. This led us down an exciting road of discovery that showed in humans, SHBG gene expression in the prostate and in the LNCaP cell line is highly complex. Our results make it apparent that the current view of human SHBG gene structure, transcription, and, perhaps, protein expression requires modification. Thus, our work has greatly opened up the possibility of studies of SHBG gene expression at its most basic levels.

Whereas when we started this study, only four human SHBG transcripts had been reported, we identified a total of at least 21 unique human SHBG gene transcripts. To our knowledge, 17 of these 21 SHBG gene transcripts are novel, with smaller sized SHBG mRNAs resulting from the alternative splicing of exons 4, 5, 6, and/or 7. In addition to the previously described promoters, P_L, and P_T, we provide evidence that the human SHBG gene utilizes a novel upstream promoter, P_N. Human SHBG gene expression varied among the four tissues that were tested in this study, suggesting they exert different controls over promoter activity, alternative splicing, and/or mRNA stability. How the expression of these transcripts is regulated, and whether the levels at which they are expressed are sufficient for biologic activity will require further study. These findings raise new and important questions regarding the regulation of human SHBG gene transcription. In confirming that human prostate expresses SHBG at the mRNA level, our results also raise questions regarding the identity of immunoreactive SHBG species previously detected in these tissues. Interestingly, we found that the LNCaP, MCF-7, and HepG2 cell lines display distinct differences in their overall SHBG expression patterns when compared to their parental tissues. Whether this observation implicates dysregulation of SHBG expression in prostate, breast, and/or liver cancer will also require further study.

Each tissue and cell line examined in this study, including normal prostate and breast, was found to specifically express the SHBG/ABP transcript. Indeed, we have shown that LNCaP cells can be used to produce elevated amounts of SHBG (Figure 1). In the prostate, this finding supports our original model in which locally expressed SHBG may regulate certain aspects of the cellular DHT response, perhaps by affecting R_{SHBG} signaling and by acting as an intracellular steroid buffer, thereby influencing AR activity. It has been reported that SHBG acts to actively transport steroids into cells by binding of endocytic receptor, megalin (13), though this finding has raised certain questions (14,15). Should megalin indeed serve this function in the prostate, then locally expressed SHBG could be a major player in steroid uptake in these tissues.

Along with the SHBG/ABP transcript, normal prostate tissue and LNCaP cells express multiple other transcripts. It is currently unclear whether any of these other transcripts encode stable SHBG isoforms, although three P_L derived transcripts specifically appear to warrant further detailed investigation. Sequences encoded within exon 8 appear to confer protein stability to SHBG, as an E326 SNP variant which possesses a premature termination codon within exon 8 incurs rapid degradation (16), leading to speculation that transcripts lacking exon 7 alone would encode inherently unstable isoforms because they suffer a frameshift and undergo premature termination within exon 8 (17). The three P_L derived transcripts lacking exon 4, exons 6 and 7, and exons 4, 6, and 7, retain the SHBG/ABP transcript exon 8 reading frame, and could be considered candidates to encode stable SHBG isoforms.

Smaller sized, immunoreactive SHBG species have been previously reported in humans, including that present in human sperm (18), which has yet to be fully characterized. With respect to our findings, its size is consistent with that of the predicted protein generated from the P_L derived transcript lacking exon 4, were it similarly postranslationally modified as SHBG (18). Two small immunoreactive SHBG species have also been detected by mass spectroscopy in studies on colocalization of SHBG with the oxytocin receptor (19,20). If alternatively spliced SHBG transcripts do encode stable SHBG isoforms, it is possible that their functions would differ, and perhaps even compete with SHBG. Exon 4 encodes amino acid residues known to be involved in hormone binding and homodimerization. Stable isoforms derived from transcripts that lack exon 4 would probably have these properties tempered or eliminated. It is interesting that all of the P_L derived alternatively spliced transcripts retain the signal peptide and the R_{SHBG} binding motif within exon 3. If these domains retain their functions in related SHBG isoforms, a scenario could be envisioned whereby they act to regulate R_{SHBG} signaling. Further study is needed to address whether additional SHBG isoforms are stably expressed, and if so, to determine their biologic properties, especially with regard to steroid binding and to R_{SHBG} signaling.

Based on the primary nucleotide sequences of exons 1N and 1T, it appears unlikely that in humans, any transcripts derived from either P_N or P_T are translated into stable SHBG isoforms. The first potential ATG start codon encountered within exon 1N occurs at the beginning of a short, four amino acid open reading frame. The succeeding ATG codon lies at the beginning of a 32 amino acid open reading frame, which, were it not interrupted by a stop codon, would merge into the SHBG/ABP reading frame in exon 2. Thus, it is unlikely that P_N transcripts encode stable and functional translation products. The potential biologic significance of transcripts originating from P_T and containing exon 1T remain unclear, especially given the exon 1T sequence that we obtained in this study. We are presently confirming whether the A insertion we observe at position 6 of exon 1T sequence is indeed a polymorphism. If so, P_T derived transcripts containing this sequence would be predicted to encode a 40 amino acid long protein with no similarity to SHBG/ABP. P_T derived transcripts that do not contain this insertion would instead encounter the first potential ATG start codon within exon 2. This is followed by a short open reading frame of only nine amino acids. Studies in transgenic mice have led to

suggestions that an ATG start codon located further downstream within exon 2, or that an unconventional start codon may be utilized by this transcript, but such evidence remains to be obtained in human cells. In lieu of such evidence, we speculate that in humans, P_T derived transcripts lacking the insertion at position six of exon 1 are inconsequential at the protein level.

The appearance of such transcripts fits a larger picture in which the mammalian SHBG gene is in a state of evolutionary flux. The core human SHBG gene module contains the promoter, P_L, exon 1L, and downstream exons 2-8, and is responsible for expression of the SHBG/ABP transcript. The human SHBG gene shares or utilizes additional promoter elements located within two adjacent genes on chromosome 17p13.1 that are transcribed in an opposite orientation. P_T, is located 1.4kb upstream of P_L, and shares regulatory elements with the adjacent polyamine N-acetyltransferase (SAT2, NM_133491) gene. And, based on our chromosomal mapping of SHBG exon 1N to a site 16.0kb upstream of P_L, P_N is probably located within intron 1 of the fragile X mental retardation related 2 (FXR2, NM_004860) gene. Such overlapping transcriptional units and bi-directional promoter arrangements are far from unique, they have been described for a number of genes, and computational studies of gene organization predict them to occur frequently within the human genome (21, 22)**.** As a side note, our discovery of P_N, and the prior discovery of P_T points out a drawback in such theoretical computational studies based on small intervals (~1kb) between adjacent genes, namely that structural and regulatory factors are as, if not more important than intergenic distance. Thus, human P_N and P_T sequences are likely to have been hijacked from adjacent genes, and P_N and P_T derived transcripts probably arise as a consequence of positional effects.

Consistent with the idea that in mammals, the SHBG gene is in evolutionary flux, the rat appears to utilize different FXR2 gene sequences in the generation of its alternative SHBG transcripts. While the rat possesses homologous exon 1N sequences (Figure 7), these have yet to be reported in mature SHBG transcripts. Instead, elements within the rat FXR2 promoter itself appear to work bidirectionally. It had been speculated that human SHBG transcripts might also originate from the FXR2 gene locus based on the sequence homology between the human and rat FXR2 promoter regions (23). Our RACE assays gave no evidence that the human exon 1A homologue is incorporated into alternative human SHBG transcripts in LNCaP, MCF-7, or normal testis, although this does not conclusively rule out low activity or tissue specific expression. Furthermore, in humans, an "ATT" is found at the position corresponding to the putative rat exon 1A ATG start codon, and no other potential ATG start codons are found within this region. And, were human exon 1A were spliced directly to exon 2 as is the case in the rat, we speculate that, similar to exon 1T containing transcripts, such transcripts would not code functional proteins.

The combined qPCR analysis and RT-PCR profiling in this study revealed differences in SHBG transcript expression between LNCaP, MCF-7, and HepG2 cells. Specifically, relative to normal liver tissue, HepG2 cells were found to express lower levels of the SHBG/ABP transcript and increased levels of alternatively spliced transcripts.

Furthermore, P_N derived transcripts were undetectable in normal liver, while they were detectable, albeit with low abundance, in HepG2 cells.

Finally, we performed microarray analysis of LNCaP cells, LNCaP cells that were induced to express elevated amounts of SHBG, and induced LNCaP cells that were also treated with DHT. Our early data analysis suggests that SHBG expression can influence LNCaP cells in at least three distinct ways. First, SHBG alone may have effects on gene expression. Second, SHBG may influence genes which are activated or repressed by the AR. And third, consistent with the model that serves as the basis for this proposal, in the presence of DHT, SHBG can affect the expression of genes that are not responsive to DHT alone. This observation is consistent with genes responding to an activated R_{SHBG} pathway. Furthermore, this is also consistent with our speculation that SHBG expression is functionally altered in prostate cancer. Now that we have established a basic foundation for SHBG expression in normal prostate tissue, our microarray results will be very important for establishing biologic significance to SHBG expression. Should these results confirm our initial findings that suggest SHBG indeed acts to regulate biologic signaling in the prostate, a detailed study to ascertain the effects of SHBG regulation in prostate cancer will be warranted.

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APPENDICES:

Figure 1. SHBG overexpression is induced by PonA in L5S2 cells

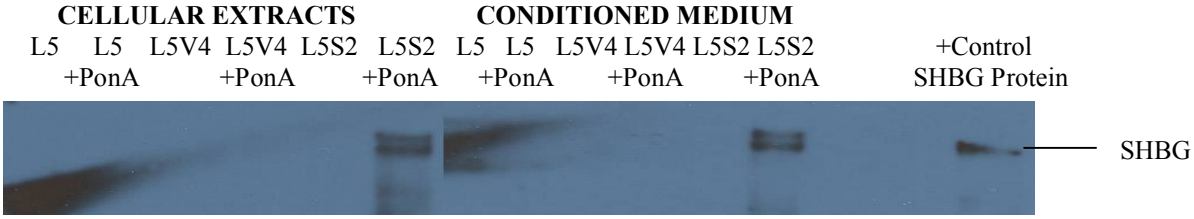


Figure 1: PonA induces SHBG protein expression in LNCaP cells stably transfected with pVgRXR (a plasmid encoding the PonA-activatable transactivator), and the inducible construct, pIND/Hygro/SHBGsense (a plasmid that expresses the secreted form of SHBG in response to the activated transactivator). Parental L5, vector control L5V4, and inducible L5S2 cells were plated in duplicate in 6 well dishes at 75% confluence. After incubating for 48 hours, cells in one well were exposed to 10uM PonA for 24 hours, while control, unexposed cells were mock treated with solvent. Total cellular protein was prepared, and conditioned medium was isolated and spun down to remove cells and cellular debris. Cellular extracts and 30ul aliquots of conditioned medium were analyzed by Western blot. 10ng of purified SHBG was loaded in the positive control lane. This short exposure shows inducible expression of SHBG in L5S2 cells, and secretion. Two SHBG bands are visible in the induced L5S2 lanes, similar findings were obtained using a constitutively expressed SHBG full length construct in LNCaP cells (see Figure 2). The reason for this observation is unclear at the present time.

Figure 2. Western blot and immunofluorescence analysis of SHBG overexpression in LNCaP clonal cell lines

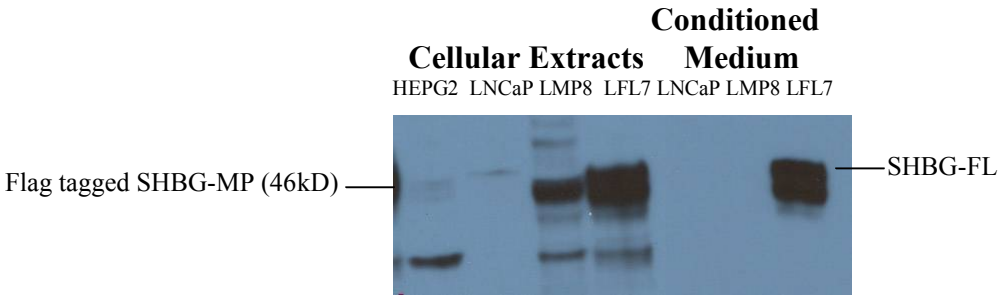


Figure 2: Ectopic expression of Flag-tagged SHBG-MP and Flag-tagged SHBG-FL in stable LNCaP clones. Total cellular protein extracts were prepared from parental LNCaP cells, and clonal LMP8 and LFL7 cells (transfected with the FLAG tagged SHBG MP and FL constructs, respectively), as well HEPG2 cells. Conditioned medium was isolated from parental LNCaP and clonal LMP8 and LFL7 cells, and centrifuged to remove cells and cellular debris. Ectopically expressed and secreted SHBG was detected by Western blot analysis, shown above, using the anti-human SHBG WAK-S102-12-53 antibody.

Figure 3. Immunohistochemical detection of SHBG in inducible LNCaP cells-steroid effects on localization

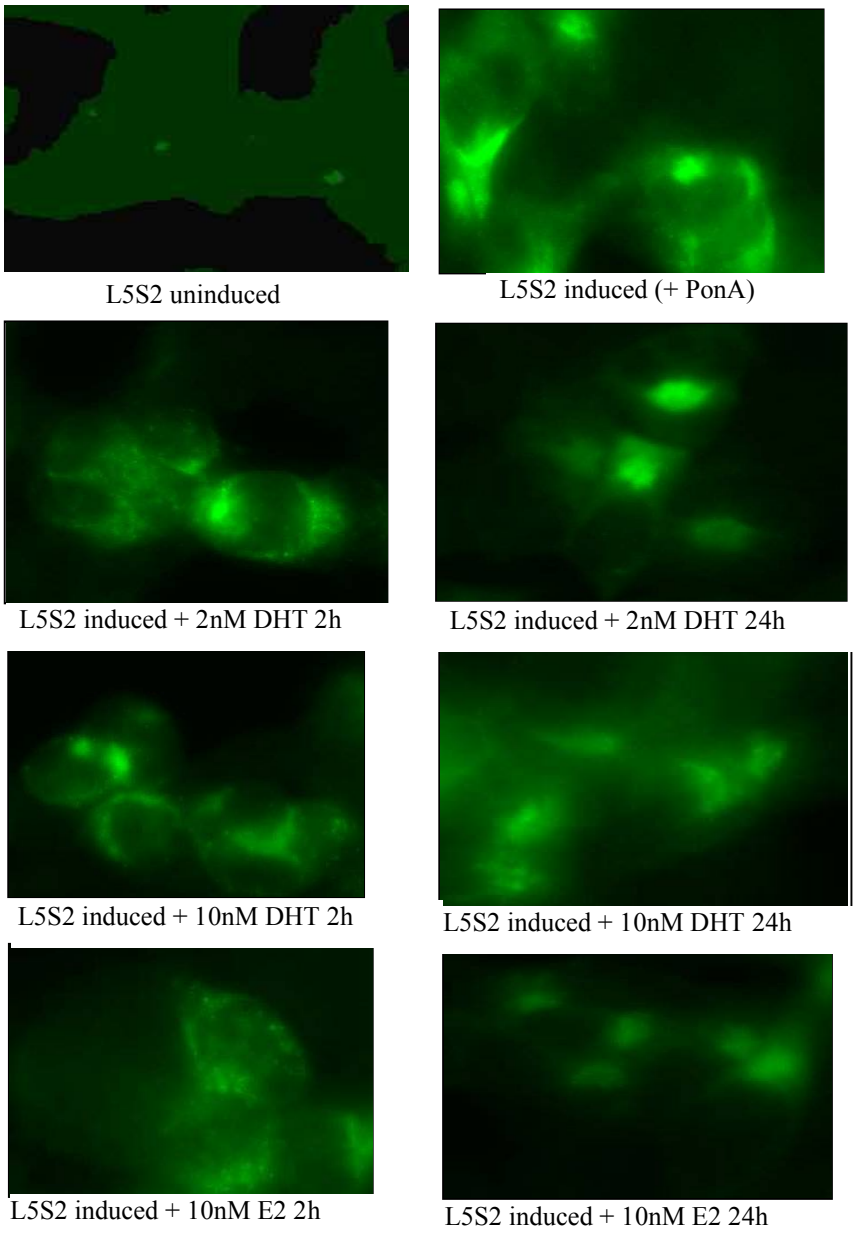


Figure 3. Immunohistochemical detection of SHBG in inducible L5S2 cells, and effects of DHT and E2 treatment. L5S2 cells were plated on glass slides, induced with Pon A (where indicated), treated with the indicated steroid for either 2 or 24 hours, and then fixed, and exposed to an anti-human SHBG polyclonal antibody. They were developed with a rabbit anti-mouse IgG1 linked to the green fluor, Alexa-488. Relative SHBG staining is shown (control cells exhibited very low level staining). DHT treated cells displayed a rounder morphology with a more focal SHBG staining. Cells not treated with steroid, and E2 treated cells displayed a slightly stronger, cytoplasmic staining.

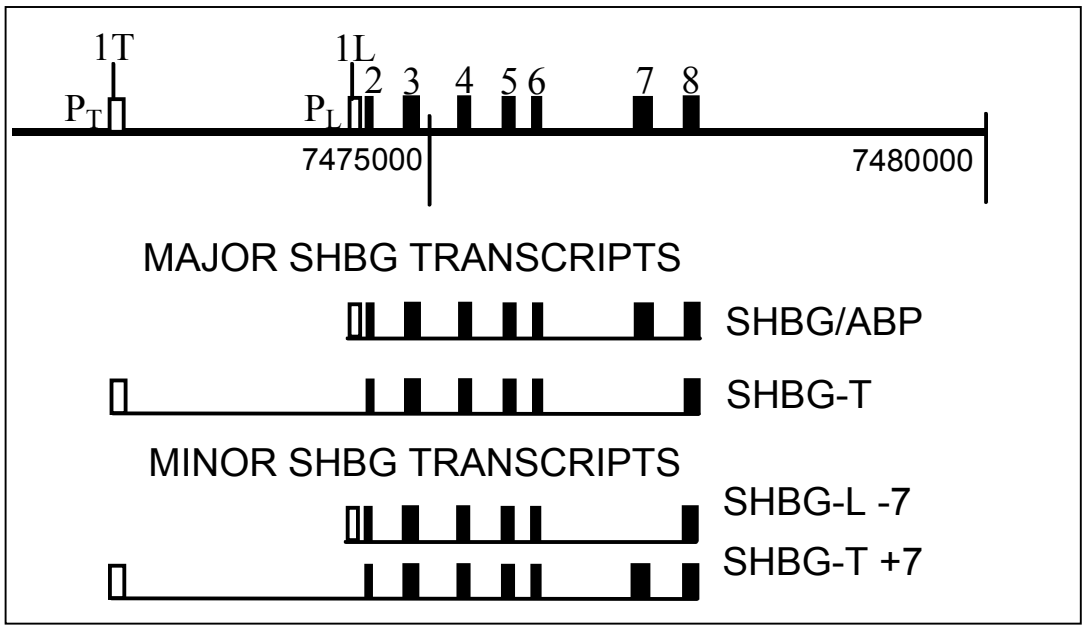


Figure 4. Structures of the four major and minor human SHBG gene transcripts reported prior to the beginning of this study. Above is a depiction of the positioning of the human SHBG gene on chromosome 17p13.1. Shown are the two promoters, P_L and P_T, the two first exons, 1L and 1T, and the downstream exons, as reported at the onset of this study. Numbers refer to relative positions on chromosome 17. Below are structures of the SHBG/ABP transcript the major testis transcript (SHBG-T), and the two alternatively spliced minor transcripts.

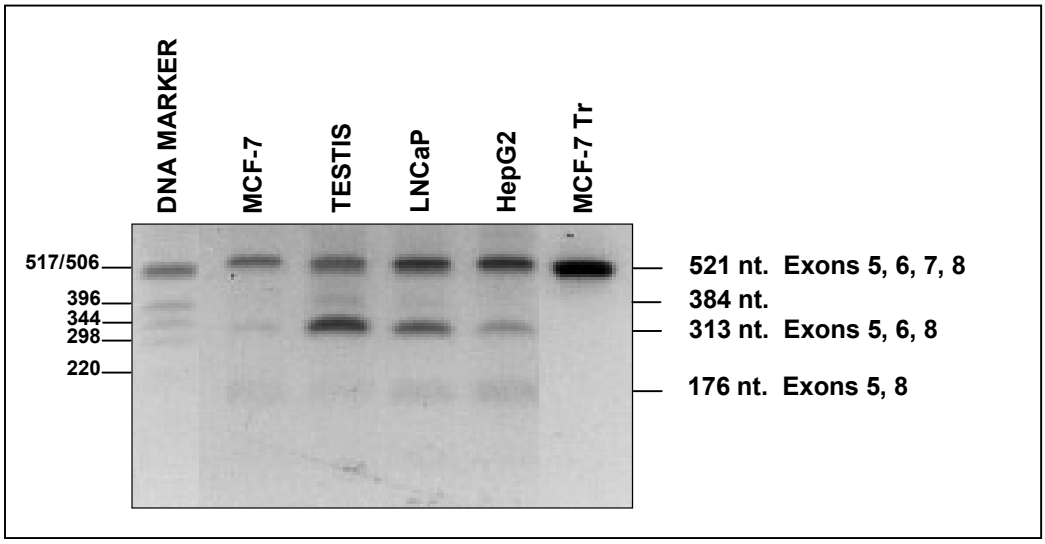


Figure 5. RT-PCR analysis of exon 5-8 containing transcripts in MCF-7, LNCaP, HepG2 cells and normal human testis tissue. Left: Total cellular mRNA was prepared from exponentially growing human MCF-7 breast, LNCaP prostate, HepG2 liver (a cell line that secretes SHBG and was included as a positive control for $SHBG_L$), and normal testis tissue (T)(expected to express higher amounts of $SHBG_T$ lacking exon 7). First strand cDNAs were generated using an oligo dT primer. The SHBG primers, SHBG exon5 forward: 5'-ACTCAGGCAGAATTCAATCTC-3' and SHBG exon8 reverse: 5'-CTTTAATGGGAAGCGTCAGT-3', flanked exon 7 and were expected to direct amplification of a larger 521 bp $SHBG_L$ fragment and a smaller 313 bp $SHBG_T$ fragment in the same reaction. Products were electrophoresed through a 1% agarose gel. Three RT-PCR transcripts were generated in the MCF-7 and LNCaP lanes, and four in the HepG2 and testis lanes. M_{Tr} is an MCF-7 clone that was stably transfected with a plasmid that constitutively expresses high amounts of $SHBG_L$. Bands were reamplified and sequenced. SHBG RT-PCR transcript structures are given on the right.

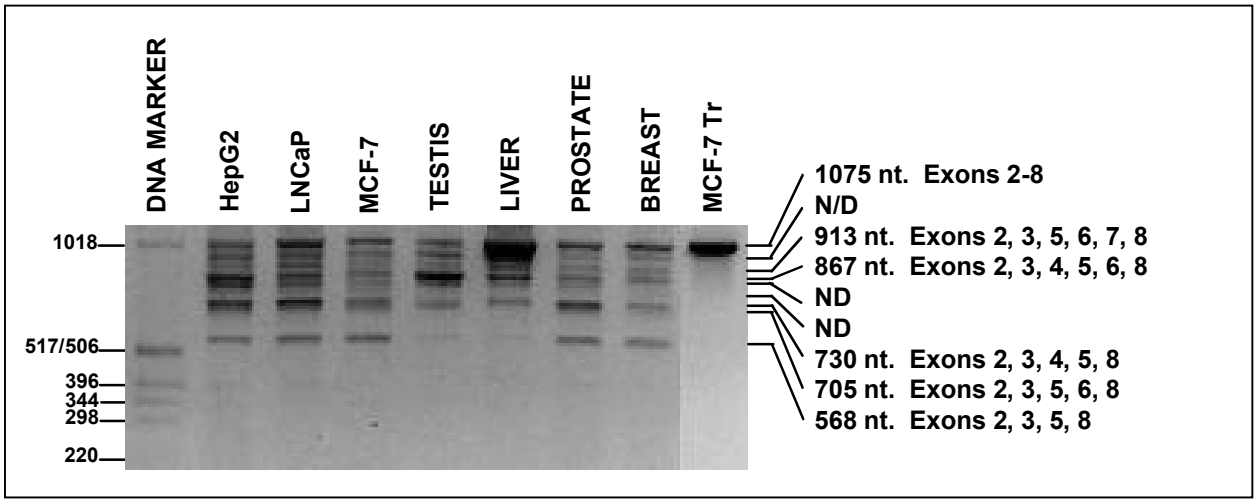


Figure 6. RT-PCR analysis of exon 2-8 containing transcripts in HepG2, LNCaP, MCF-7 and normal human testis, liver, prostate and breast tissues. PCR primers, specific for exon 2 and exon 8 were used to amplify cDNAs prepared from the indicated samples. Products were electrophoresed through a 1% agarose gel. Where possible, bands were reamplified and sequenced. RT-PCR fragment transcript structures are given on the right. ND- not done, bands did not reamplify.

RT-PCR ASSAY Exon 2-8		SAMPLE	HepG2	LNCaP	MCF-7	Testis	Liver	Prostate	Breast
RT-PCR Fragment Structure	size								
Exons 2-8	1075		+	+	+	+	**++++**	+	+
Unknown #1			+	+	+	+	+	+	+
- Exon 4	913		+	+	+	+	+	+	+
- Exon 7	867		++	+	+	++	+	+	+
Unknown #2			++	+	+	++	+	+	+/-
Unknown #3			+	+	+	-	-	+	+
- Exons 6,7	730		+	+	+	+/-	+	+	+
- Exons 4,7	605		+/-	+/-	+/-	+/-	-	+/-	+/-
- Exons 4,6,7	468		+	+	+	+/-	+/-	+	+

RT-PCR ASSAY Exon 1L-8		SAMPLE	HepG2	LNCaP	MCF-7	Testis	Liver	Prostate	Breast
RT-PCR Fragment Structure	size								
Exons 1L-8 (SHBG/ABP)	1130		++	+	++	++	**++++**	+/-	+
Unknown #1			+	+/-	+/-	++	+++	-	-
- Exon 4	968		+	+/-	+	++	++	-	-
- Exon 7	922		++	+	+	++	++	+/-	-
Unknown #2			++	-	-	+	+	-	-
- Exons 6,7	785		++	+	-	+	+	+/-	-
- Exons 4,7	760		+/-	-	-	-	-	-	-
- Exons 4,6,7	623			-	-	-		-	-
- Exons 4,5,6,7	463		+	-	-	+	+	-	-

RT-PCR ASSAY Exon 1T-8		SAMPLE	HepG2	LNCaP	MCF-7	Testis	Liver	Prostate	Breast
RT-PCR Fragment Structure	size								
Exons 1T-8	1151		+/-	+	+/-	++	+	+	+
Unknown #1			-	+/-	-	?	+/-	-	-
- Exon 4	989		+/-?	+/-	+/-?	?	+/-	+/-	?
- Exon 7	943		+/-?	+	+/-?	++++	+	+/-?	+/-?
Unknown #2			+/-?	+	+/-?	?	-	+/-?	-
- Exons 6,7	806		+/-	+	+	?	+	+	+/-?
- Exons 4,7	781		+/-?	+/-	-	+	-	-?	+/-?
- Exons 4,6,7	644		+	+	+	+/-	-	+/-	+/-
- Exons 4,5,6,7	484		+/-	+	+	-	-	-	-

RT-PCR ASSAY Exon 1N-8		SAMPLE	HepG2	LNCaP	MCF-7	Testis	Liver	Prostate	Breast
RT-PCR Fragment Structure	size								
Exons 1N-8	1146		-	++	+	+/-	-	+	-
- Exon 4	984		+/-?	++?	-	+?	-	-	-
- Exon 7	938		-	++?	-	+?	-	-	-
- Exons 6,7	801		+/-?	++?	-	-	-	-	-
- Exons 4,7	776		-	++?	-	-	-	-	-
- Exons 4,6,7	639		-	+	-	-	-	-	-
Unknown			-	+	-	-	-	-	-
- Exons 4,5,6,7	479		-	?	-	-	-	-	-

Table 1. Table of RT-PCR results. Summary of results from the exon 2-8 RT-PCR assay, and from the three individual exon 1-8 RT-PCR assays. From left to right- Assay titles, RT-PCR fragment structures, RT-PCR fragment sizes, and the samples analyzed in this study. Relative amounts of each RT-PCR fragments as they appeared in the assays are denoted by the following- "-" not detectable, "+/-" barely detectable, "+" detectable, … "++++" most abundant. A "?" denotes that the RT-PCR fragments could not be fully characterized due to positional effects- this is due to either neighboring bands that are highly intense, or an inability to distinguish between similarly sized fragments. These bands are in the process of being reproduced and reamplified for sequencing to determine their exact identities.

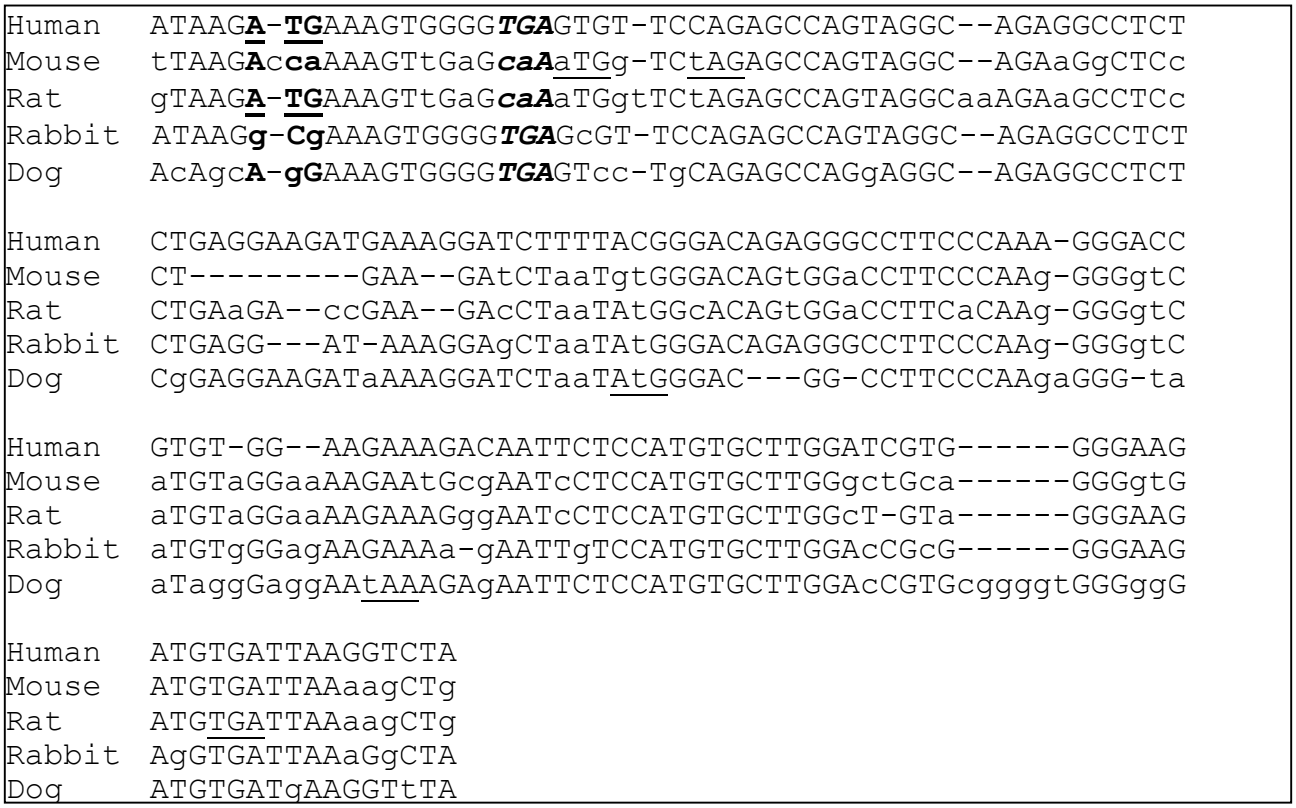


Figure 7. Sequence of human SHBG exon 1N. Shown is an alignment between the full human SHBG exon 1N sequence and corresponding sequences from mouse, rat, rabbit and dog. The putative human ATG start codon is in bold and underlined. The TGA stop codon is in bold and italics. Conserved nucleotides between species are capitalized, differences are in lower case letters. The human exon 1N open reading frame is predicted to be only 3 amino acids long.

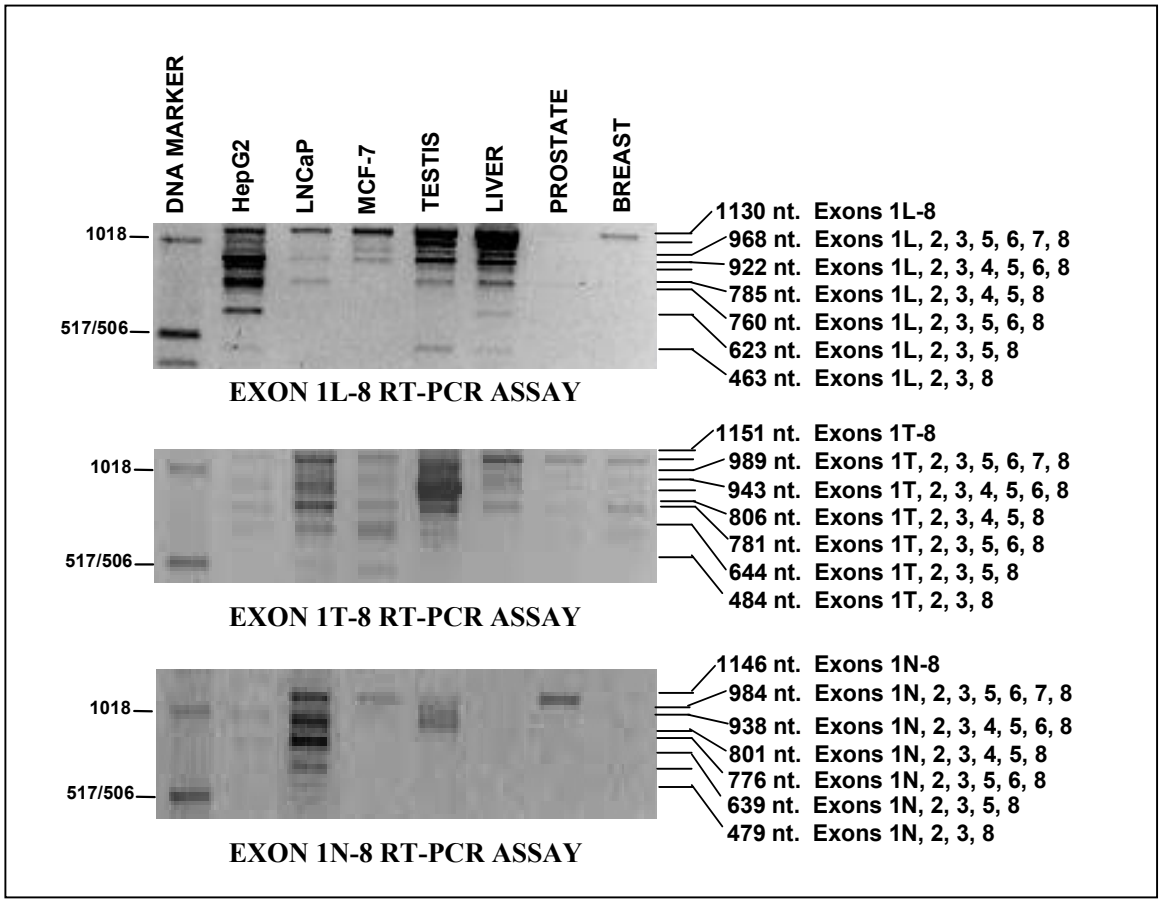


Figure 8. RT-PCR analysis of transcripts including exon 1L (top), exon 1T (middle), and exon 1N (bottom). Separate individual RT-PCR amplifications were performed using 5' primers specific for exon 1L, 1T, and 1N, and a common exon 8 3' primer. Products were electrophoresed through a 1% agarose gel. Where possible, bands were reamplified and sequenced. RT-PCR fragment transcript structures are given on the right. Bands without exon structures were unable to be reamplified.

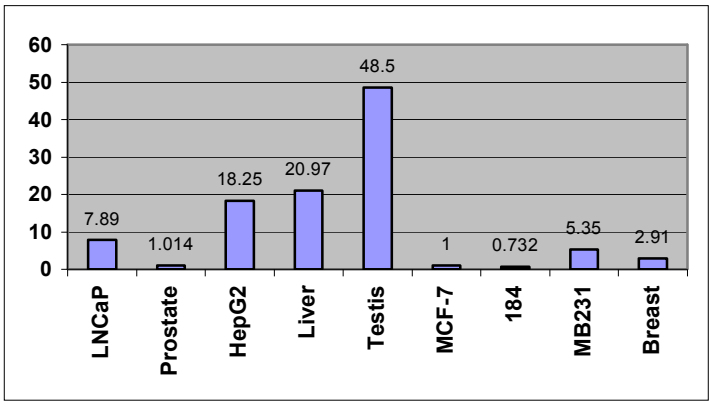


Figure 9A- Relative overall SHBG expression levels in human cell lines and tissues. Quantitative RT-PCR was performed using primers specific for conserved SHBG transcript regions within exons 2 and 3. Average expression levels are given above the data bars, and are normalized to MCF-7 expression levels. Experiments were performed in triplicate, on either two or three RNA samples.

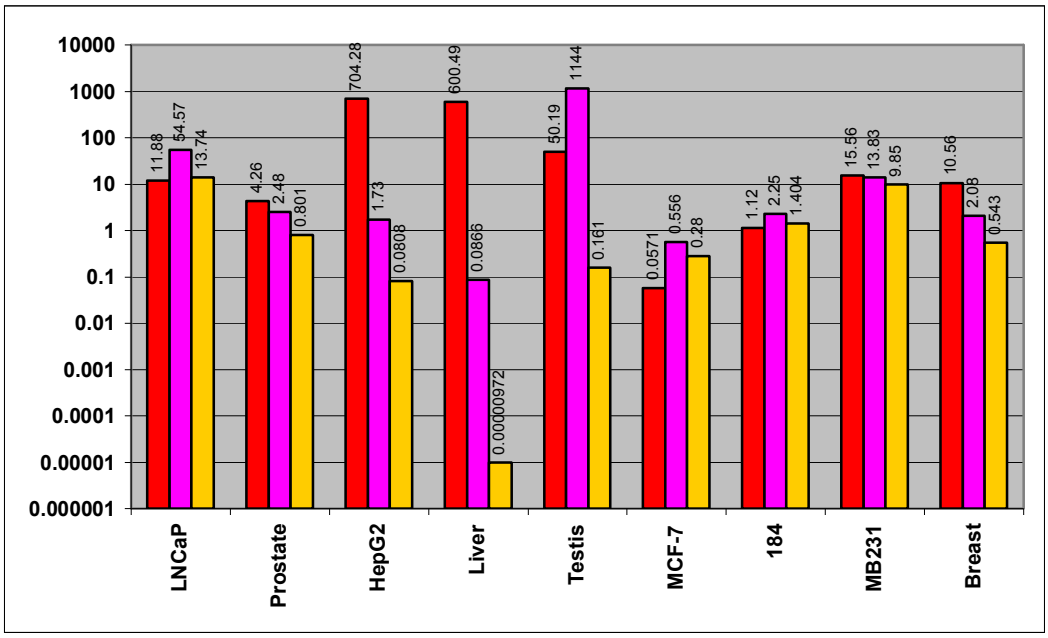


Figure 9B SHBG gene promoter utilization. Quantitative RT-PCR was performed on human cell lines and tissues to determine relative expression of SHBG gene transcripts originating from the downstream promoter (PL, red), the intermediate promoter (PT, pink), and the novel upstream promoter (PN, orange). Primer sets were specific for SHBG exons 1L and 2, 1T and 2, and 1N and 2, respectively. Experiments were performed in triplicate, on either two or three RNA samples. Expression levels are normalized to overall MCF-7 expression levels from Figure 9A. Note- this is a logarithmic scale. Expression levels are denoted above each data bar.

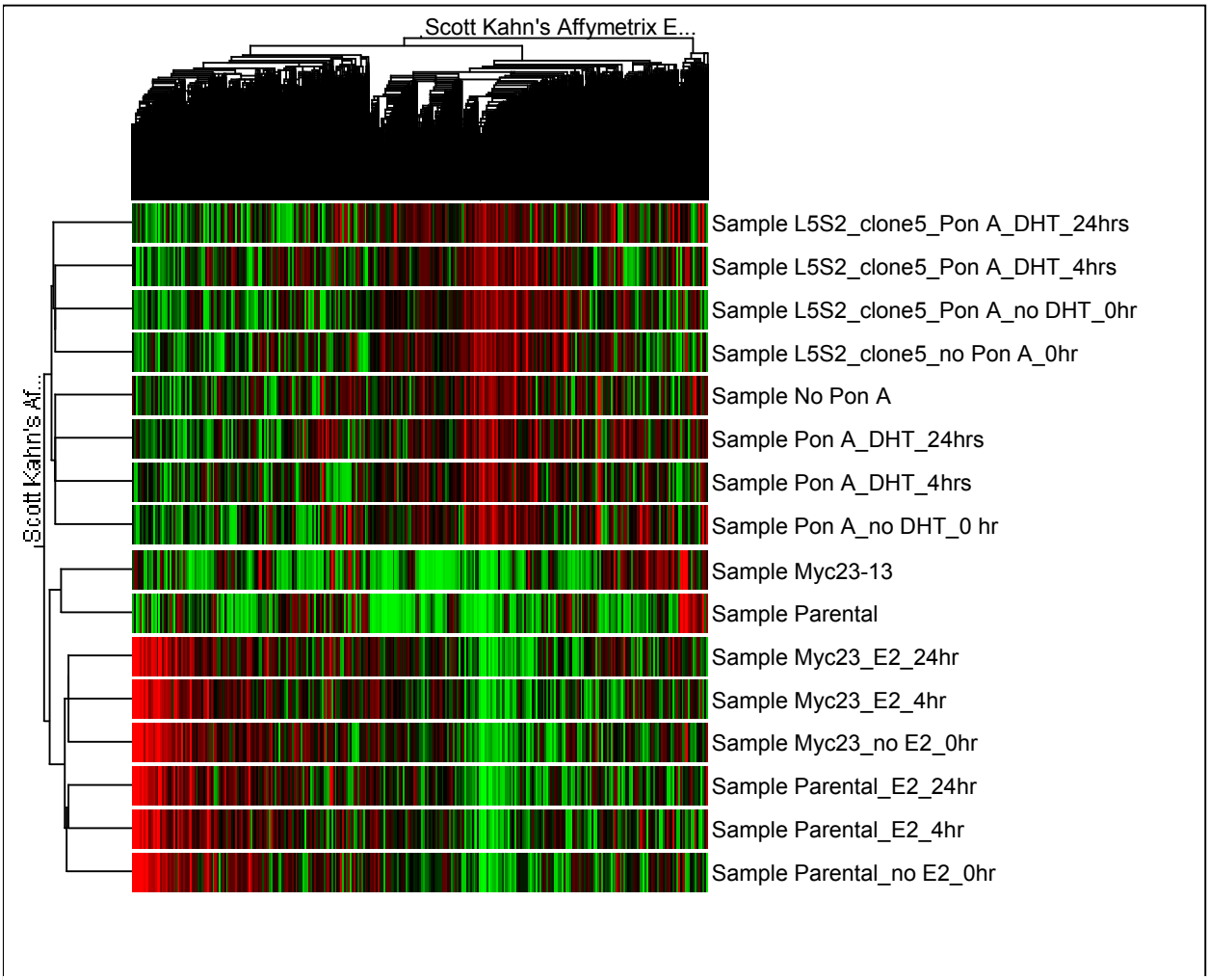
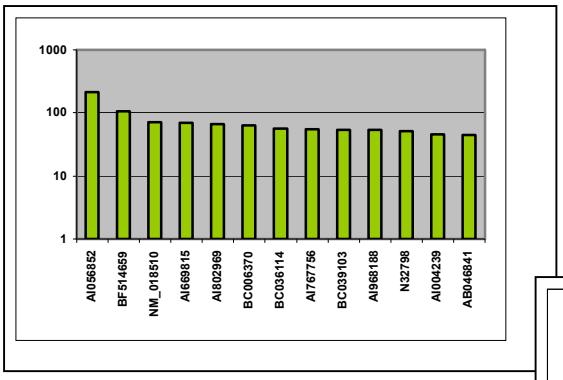


Figure 10. Similarity Diagram of Cell Lines and Treatment Conditions Based on Gene Expression Profiles. Sixteen different LNCaP, HepG2, and MCF-7 samples were analyzed by microarray analysis. Effects of SHBG induction in LNCaP cells were investigated using the inducible LNCaP cell line, L5S2. Total cellular RNA was prepared from L5S2 cells, and its sister vector control cell line, L5V4, as well as from cells that had been treated with the inducing agent, PonA (10uM) for 24 hours, PonA and then 10nM DHT for 4 hours, and PonA and then 10nM DHT for 24 hours. Total cellular RNA was also prepared from HepG2 liver cancer cells, and a constitutive SHBG overexpressing clone, HepG2myc23. In addition, Total cellular RNA was prepared from MCF-7 breast cancer cells and a constitutive SHBG overexpressing clone, MCF-7myc23, as well as from the same two cell lines treated with 10nM estradiol for 4 or 24 hours. Total cellular RNA was pretreated with RNase-free DNase. Following this, the samples were cellular RNAs were sent to our colleagues at the Yerkes Genomics Core Facility at Emory University. RNAs were analyzed by Agilent Bioanalyzer to check the RNA quality. Samples were labeled and hybridized to Affymetrix Human Genome U133 Plus 2.0 Array chips. Raw data was collected by GCOS software and analysis of the microarray data was performed using GeneSpring software. Shown is similarity diagram which compares the 16 different samples analyzed in this study, with respect to overall gene expression profiles.



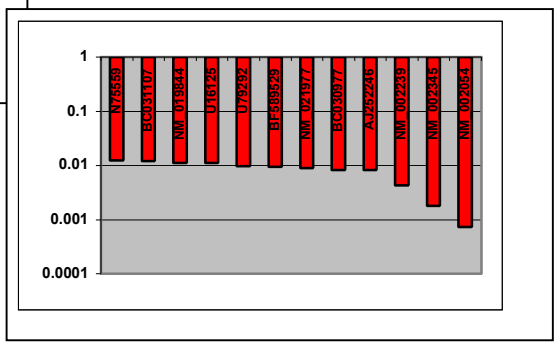
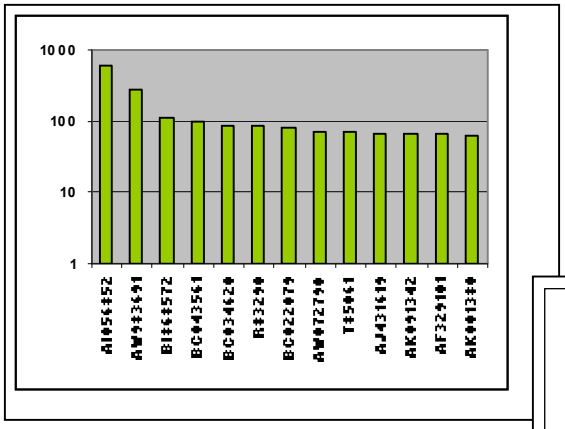


Figure 11A- Genes whose expression in L5S2 cells treated with PonA for 24 hours is increased by 44-fold or greater (green, right) or is decreased by 80-fold or greater (red, below) as compared to PonA treated vector control L5V4 cells.



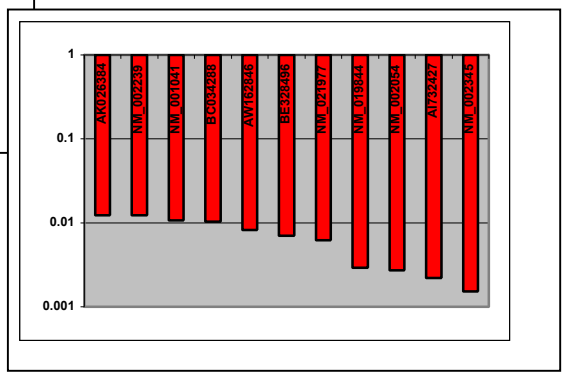


Figure 11B -Genes whose expression in L5S2 cells treated with PonA and 10nM DHT for 4 hrs is increased by 60-fold or greater (green, right) or is decreased by 80-fold or greater (red, below) compared to similarly treated vector control L5V4 cells.

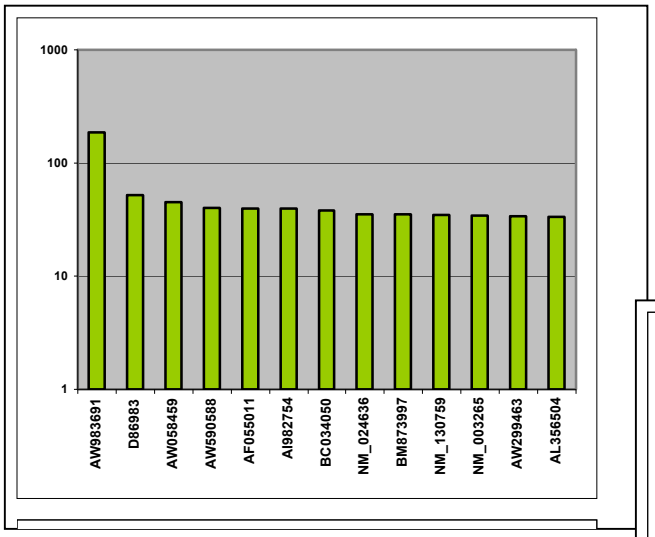
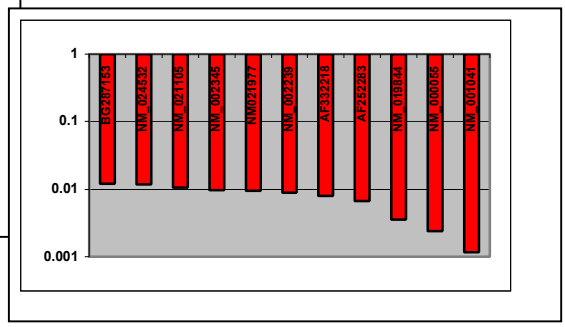


Figure 11C -Genes whose expression in L5S2 cells treated with PonA and 10nM DHT for 24 hrs is increased by 33-fold or greater (green, right) or is decreased by 80-fold or greater (red, below) compared to similarly treated vector control L5V4 cells.



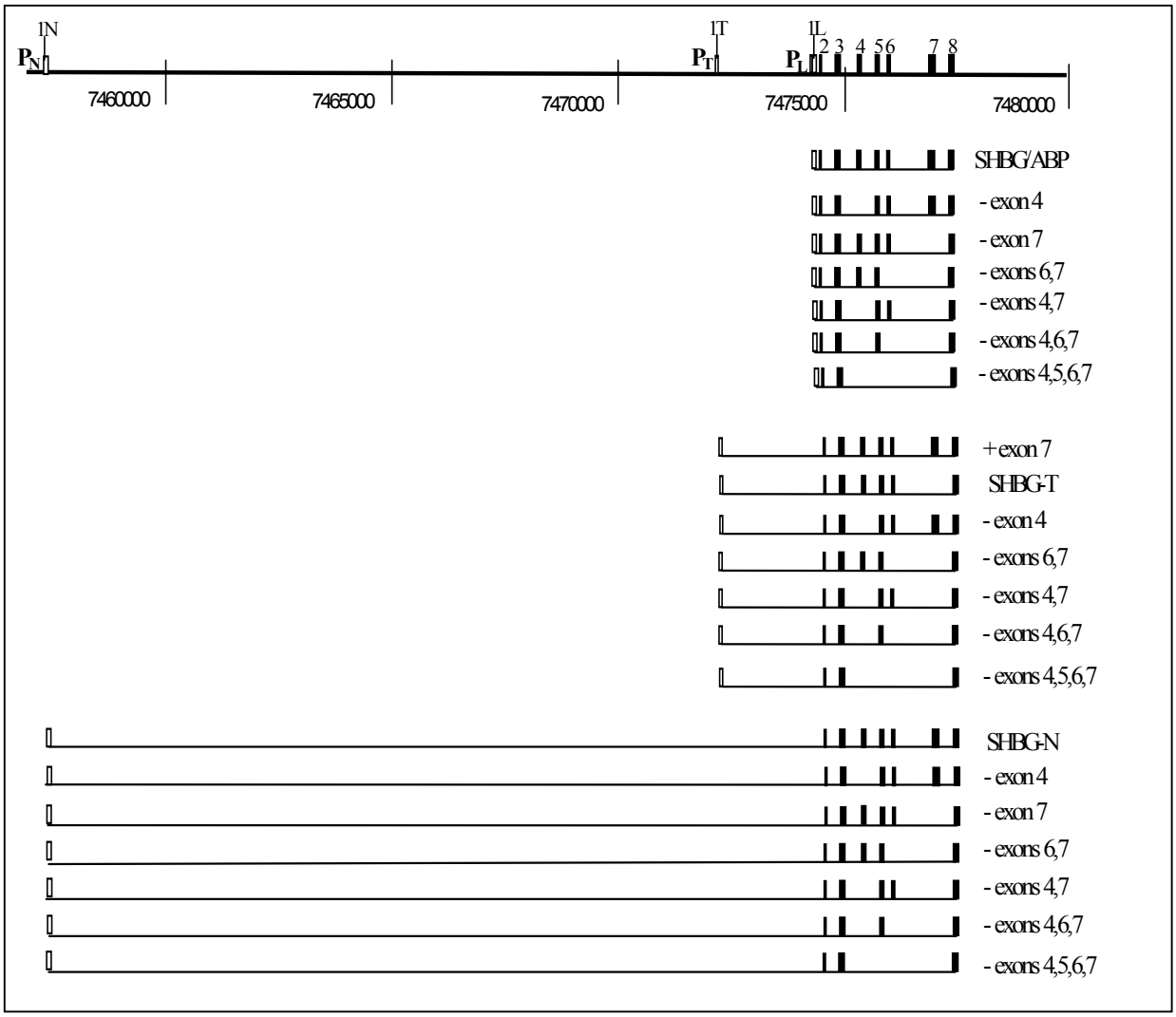


Figure 12. The newly revised profile of human SHBG gene transcription.
Shown is a comprehensive picture of the 21 human SHBG gene transcripts that we detected in this study. Above is a depiction of the positioning of the human SHBG gene on chromosome 17p13.1. Shown are the three promoters, P_L, P_T, and P_N, the three first exons, 1L, 1T, and 1N, and the downstream exons as ascertained in this study. Numbers refer to relative positions on chromosome 17. Below are structures of the 21human SHBG gene transcripts detected in this study, with their compositions given at the right.

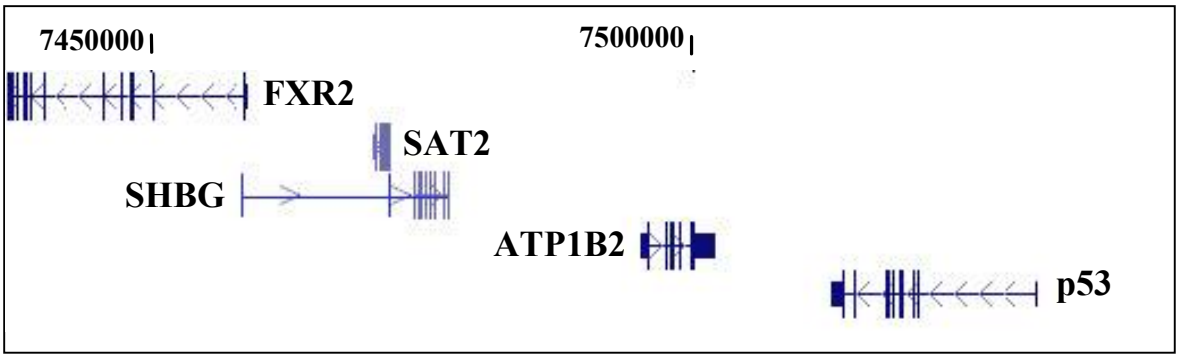


Figure 13. The human SHBG gene and neighboring genes on chromosome 17p13.1. Exon structures of the SHBG gene and its neighboring genes are given as vertical lines. Transcriptional polarity is given by arrows. Note that the polarity of the SHBG gene is opposite that of the SAT2 and FXR2 genes. SHBG upstream exon 1T abuts the SAT2 gene, and exon 1N is located within intron 1 of the FXR2 gene. Note also the close proximity of the p53 gene, implicating the SHBG gene locus in prostate and other tumors that have undergone allelic deletions of the p53 locus.

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